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(54) Title: BONE MARROW TRANSPLANTATION (57) Abstract The present invention provides a method for bone marrow transplantation from an HLA-nonmatched donor to a patient which comprises conditioning the patient under a suitable regimen followed by transplant of a very large dose of stem cells which is at least about 3-fold greater than the conventional doses used in T cell-depleted bone marrow transplantation. The patient is conditioned under lethal or supralethal conditions for the treatment of malignant or non-malignant diseases, or under sublethal conditions for the treatment of non-malignant diseases. The transplant may consist of T cell-depleted bone marrow stem cells and T cell-depleted stem cell-enriched peripheral blood cells from the HLA-nonmatched donor, preferably a relative of the patient, which donor was previously treated with a drug, e.g. a cytokine such as granulocyte colony-stimulating factor (G-CSF).		

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BONE MARROW TRANSPLANTATION

Field and Background of the Invention

The present invention relates to bone marrow transplantation in humans, particularly from HLA-nonmatched donors.

Bone marrow transplantation (BMT) is being increasingly used in humans. In genetically identical twins there are no immunological barriers to BMT, but in other circumstances genetic disparities result in immune-related complications, including graft rejection and graft-versus-host disease (GVHD) (Gale and Reisner, 1986).

GVHD can be prevented by using T-cell-depleted bone marrow. Since 1980, we and others have successfully used mismatched T-cell-depleted marrow for the treatment of children with severe combined immune deficiency (SCID) (for reviews, see O'Reilly et al., 1989; Reisner et al., 1984; Reisner, 1990). The newly formed donor-type T cells in these patients are tolerant of the host and do not induce GVHD. Based on these promising results, the use of T-cell-depleted bone marrow was extended to the treatment of leukemia patients for whom matched sibling donors were not available and to those individuals who had matched sibling donors but were nevertheless at high risk for GVHD.

However, the experience with leukemia patients was disappointing, due to a high rate of graft rejection or graft failure. In recipients of HLA-identical T-cell-depleted bone marrow, the incidence of graft rejection is about 10-15%, whereas in recipients of HLA-nonidentical T-cell-depleted marrow, the rate of rejection is about 50% (O'Reilly et al., 1985).

Bone marrow allograft rejection has been documented extensively in different animal models (Reisner, 1990; Valleria and Blazer, 1989). In the mouse it can be overcome simply by increasing the marrow inoculum. We and others have been able to produce long-term immunologically vigorous chimera with a high rate of success (Reisner, 1990). A key question, therefore, is whether the differences between T-cell-depleted transplants in mouse and man are simply due to technical discrepancies, such as the low number of pluripotent stem cells in the

human bone marrow aspirate (which is limited to the iliac crest and is highly contaminated with peripheral blood), or whether they reflect the great disparity between outbred humans as opposed to inbred mice. Recent studies carried out in our laboratory have illustrated the quantitative basis of bone marrow allograft rejection and strongly indicate that the former possibility is more likely to be true. Thus, in the mouse model, reduction of the hematological parameters typical for marrow rejection was positively correlated with several parameters reflecting the residual immunity remaining after the conditioning with lethal irradiation, and inversely correlated with the marrow inoculum size. In particular, it was demonstrated that, even in the most stringent murine models of bone marrow allograft rejection, this complexed reaction can be overcome if a sufficiently large marrow transplant is used.

More recently, advances made in the area of autologous BMT have shown that, in cancer patients receiving such transplants, treatment with granulocyte colony-stimulating factor (G-CSF) or other cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3), leads not only to elevated levels of neutrophils in the peripheral blood, but also to mobilization of pluripotential stem cells from the marrow to the blood. Thus, following induction with G-CSF, it became possible to collect by leukapheresis large numbers of stem cells (Caspar et al., 1993).

Among the diseases that can be treated with success by bone marrow transplantation, are more than 20 otherwise fatal diseases that include the six or seven genetically different forms of SCID, various forms of congenital or genetically determined hematopoietic abnormalities, combinations of these two, certain anemias, osteopetrosis, a variety of high risk leukemias and several forms of severe life-threatening aplastic anemia. These diseases include SCID autosomal recessive with and without B cells (no ADA deficiency); SCID X-linked recessive without B cells; SCID autosomal recessive with ADA deficiency; Wiskott-Aldrich syndrome; Blackfan-Diamond syndrome; Fanconi anemia; severe neutrophil dysfunction; chronic granulomatous disease of childhood; severe (Kostman-type) agranulocytosis; immunodeficiency and neutropenia of cartilage-hair hypoplasia; infantile and late onset osteopetrosis; aplastic anemia-toxic chemical, idiopathic, immunological, and genetic (non-Fanconi); acute myeloid leukemia; chronic myeloid leukemia; Burkitt lymphoma, and recurrent acute lymphatic leukemia. Other diseases that have been treated recently with BMT include metabolic storage diseases such as Gaucher's disease, hemoglobinopathies such as thalassemia, and even some solid tumors such as neuroblastoma. In addition, BMT can be carried out before transplantation of an organ, e.g. kidney, from a same donor to a patient.

More general application of allogeneic BMT for the treatment of patients with hematologic malignancies or other disorders is restricted by the availability of suitable donors. Less than 30% of patients who might benefit from transplant have genotypically HLA-identical siblings and only 3-5% have an one HLA-locus mismatched relative. In contrast, nearly all patients have an HLA-haploidentical relative (parent, child, sibling) who could serve as a donor. To date, transplantation of unmodified bone marrow from HLA-haploidentical two or three loci incompatible donors has been associated with unsuccessful outcome due to the high incidence (80%) of severe GVHD. The risk of graft failure may be 20% or higher. Extensive T cell-depletion of mismatched donor marrow can be used to effectively prevent GVHD, but the undesirable consequence of such transplants has been an increase in the incidence of graft failure to as high as 50% (O'Reilly, 1985 and 1988).

In view of the expanded approach to treatment of many severe diseases with BMT, a method for achieving high rates of engraftment of bone marrow cells from HLA-nonmatched donors, with low incidences of graft rejection and GVHD, would be highly desirable.

Summary of the Invention

It is an object of the present invention to provide a method for treatment of a patient by bone marrow transplantation which comprises transplanting to said patient, after conditioning under a suitable regimen, a very large dose of stem cells, preferably a dose at least about 3-fold greater than the doses usually used in conventional T-cell-depleted bone marrow transplantation, from an HLA-nonmatched donor.

The method of the invention is accomplished, for example, by transplanting to the host patient T-cell-depleted bone marrow cells and T-cell-depleted stem cell-enriched peripheral blood cells from an HLA-nonmatched donor, such as an HLA haploidentical "three loci" incompatible family member, said stem cells of the peripheral blood being mobilized by treatment of the donor with a suitable cytokine, e.g G-CSF.

Brief Description of the Figures

Figs. 1A-B depict curves representing cumulative proportions of patients reaching 500 (1A) and 1000 neutrophils (1B).

Detailed Description of the Invention

Graft failure is presumably a result of the immunological rejection of donor hemopoietic cells by the host residual immune system, but it could also be mediated by other mechanisms, including competition between donor and residual host stem cells for the limited available niches in the bone marrow stroma, as well as the availability of facilitating cells in the donor inoculum. In the mouse model, the immunological rejection of T cell-depleted histoincompatible BMT can be overcome by increasing radiation or by adding selective measures with minimal toxicity, such as splenic irradiation or *in vivo* treatment with anti-T monoclonal antibodies, to the conditioning regimen. Stem cell competition can be manipulated in favour of donor type cells by increasing the size of the T-cell depleted bone marrow inoculum (Reisner, 1978) or by adding myeloablative drugs (busulphan, thiotepea) to the radiation therapy (Lapidot et al., 1988; Terenzi et al., 1990).

The means of overcoming graft failure elucidated in the experimental model can be applied in the clinical setting by combining approaches which increase both the conditioning of the host and the size of the stem cell inoculum.

To this end, we designed a conditioning regimen which added anti-thymocyte globulin (ATG) and thiotepea, a powerful myeloablative agent, to cyclophosphamide and total body irradiation (TBI) in a single fraction at a fast dose rate, to enhance both immunosuppression and myeloablation. It has recently been demonstrated that the administration of recombinant human granulocyte colony-stimulating factor (rhG-CSF) or recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) can mobilize a sufficient number of peripheral blood progenitor cells (PBPC) to permit the collection of a transplant inoculum (Matsunaga et al., 1993). Infusion of these cytokine-mobilized cells has resulted in rapid marrow recovery and sustained hematopoiesis in autologous (Gianni et al., 1990) and syngeneic transplants (Weaver et al., 1993). Two cases of allogeneic PBPC transplant have also been reported (Russel et al., 1993). Moreover, studies on PBPC transplants in animals have indicated that PBPCs can provide long-term multilineage hematopoiesis (Molineux et al., 1990).

Therefore, we attempted to increase the overall number of colony forming units-granulocyte/macrophage (CFU-GM) infused into the recipients by an order of magnitude by adding to the T cell-depleted bone marrow, peripheral blood progenitor cells obtained from the donor after the administration of rhG-CSF. These cells were subjected to the same T cell-depletion procedure. No post-grafting immunosuppressive treatment was given.

According to the invention the host patient is conditioned prior to the transplantation of

stem cells. Conditioning may be carried out under sublethal, lethal or supralethal conditions, for example by total body irradiation (TBI) and/or by treatment with myeloablative and immunosuppressive agents. According to standard protocols, a lethal dose of irradiation is within the range of 7-9,5 Gy TBI, a sublethal dose is within the range of 3-7 Gy TBI and a supralethal dose is within the range of 9,5-16 Gy TBI.

Several protocols are known for conditioning of the host patient with a myeloablative and an immunosuppressive agent without TBI. As an example, busulphan is administered from day -8 to day -5 at a daily dose of 1-2 mg/kg (sublethal), 2-5 mg/kg (lethal) or >5 mg/kg (supralethal), followed by 50 mg/kg cyclophosphamide daily in the three regimens from day -4 to day -1.

Usually, lethal and supralethal conditioning are used to treat patients having malignant diseases, e.g. various leukemias. When the patient is suffering from non-malignant diseases, e.g. various anemias, sublethal conditioning is used. Thus, when TBI is carried out, the patient is irradiated with a single or fractionated dose within the range of 7-16 Gy, preferably 7-10 Gy, most preferably 8 Gy, when lethal or supralethal irradiation is desired, and with a single or fractionated dose within the range of 3-7 Gy, preferably 6.5 Gy, when sublethal irradiation is desired. The fractionated dose may be administered during 1 to 7 days, once or 2-10 times daily.

Any immunosuppressive agent used in transplantation to control the rejection, or a combination of such agents, can be used according to the invention, such as prednisone, methyl prednisolone, azathioprine, cyclophosphamide, cyclosporine, monoclonal antibodies against T-cells, e.g. OKT3, and antisera to human lymphocytes (antilymphocyte globulin - ALS) or to thymus cells (antithymocyte globulin - ATG). Examples of myeloablative agents that can be used according to the invention are busulphan, dimethyl myleran and thiotepea.

In a preferred embodiment of the invention, the patients are treated with a combination of ATG, thiotepea and cyclophosphamide, after TBI.

The HLA-nonmatched donor may be an unrelated person to the family, but preferably will be a very close relative, most preferably a family member of the patient, such as one of the parents, a brother or a sister of the patient.

Bone marrow from the donor is obtained by aspiration of marrow from the iliac crest. T-cell depletion of bone marrow may be carried out by any known technique, for example, by soybean agglutination and E-rosetting with sheep red blood cells as described (Reisner et al., 1981, 1986).

Peripheral blood stem cells are obtained after stimulation of the donor with a single or several doses of a suitable cytokine, such as granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3). In a preferred embodiment of the invention, the donor is stimulated with G-CSF (Caspar et al., 1993).

In order to harvest desirable amounts of stem cells from the peripheral blood cells, leukapheresis is performed by conventional techniques (Caspar et al., 1993) and the final product is tested for mononuclear cells. T-cell depletion is carried out as for bone marrow and the final product is filtered and irradiated (15-40 Gy) before infusion.

The total dose of T-cell-depleted stem cells from bone marrow and peripheral blood that is to be administered according to the invention is at least 3-fold greater than conventional doses used in T-cell-depleted bone marrow transplantation. These conventional doses are within the range of 5×10^8 - 3×10^9 T-cell-depleted bone marrow cells. According to the invention, when T cell depletion is carried out by soybean agglutination and E-rosetting with sheep red blood cells, the range of mononuclear cells (MNC) in bone marrow is between of 0.1 - 0.5×10^8 /kg recipient, comprising an average of about 1.9×10^6 /kg recipient of CD34+ stem cells. Still according to the invention, the total number of CD34+ stem cells that is to be administered to the patient is increased up to at least about 3 times the above amount of CD34+ cells in the T cell-depleted bone marrow. i.e. at least about 5.5×10^6 /kg CD34+ cells. This can be achieved, for example, by the addition of T-cell-depleted stem cell-enriched peripheral blood cells to the T cell-depleted bone marrow cells, or by transplantation only of T cell-depleted stem cell-enriched peripheral blood cells in the desired high dose. The mobilization of the peripheral blood stem cells is done, for example, by stimulation of the donor with a suitable drug, e.g. a cytokine.

The method of the invention is suitable for the treatment of diseases curable by bone marrow transplantation, including leukemias, such as acute lymphoblastic leukemia (ALL), acute nonlymphoblastic leukemia (ANLL), acute myelocytic leukemia (AML) and chronic myelocytic leukemia (CML), severe combined immunodeficiency syndromes (SCID), osteopetrosis, aplastic anemia, Gaucher's disease, thalassemia and other congenital or genetically-determined hematopoietic abnormalities. In addition it can be used for BMT prior to the transplantation of an organ, e.g. kidney, from the same donor to the same recipient.

The invention will now be illustrated by the following non-limiting examples.

Example 1 : Engraftment of T cell-depleted haploidentical "three loci" incompatible transplants in leukemia patients

Patients

Seventeen patients (15 male, 2 female), median age 23.2 years (range 6-51), with advanced chemoresistant leukemia (5 AML, 9 ALL, 3 CML in blastic phase) were transplanted during a period of about 9 months. All patients received grafts from HLA haploidentical "three loci" incompatible (or mismatched) family members. 6 donors were siblings, 11 were parents. Anti-donor lymphocyte antibodies, as assayed in a complement-dependent microcytotoxicity crossmatch test were not detected in any of the 12 evaluated cases. The aforementioned is summarized in Table I below which sets forth the patient's characteristics and donor-host relationship.

Table I
Patient's characteristics and Donor-Host relationship

UPN	PATIENTS			DONORS		HLA-ANALYSIS	
	Age/ Sex	Disease	Status	Age/ Sex	Relationship	Donor	Host
306	22/M	AML	REL	45/M	Father	A2B35DR4 A11B7DR2	A2B35DR4 A26B49DR6
313	37/M	AML	REL	67/F	Mother	A2B35DR11 A30B7DR2	A2B35DR11 A1B8DR3
315	17/M	ALL	REL	40/F	Mother	A2B44DR17 BLB8DR4	A2B44DR17 A2B18DR4
317	31/F	ALL	REL	39/M	Brother	A24B18DR2 A2BLDR5	A24B18DR2 A11BLDR7
319	15/M	CML	BT	49/F	Mother	A2B35DR3 A3B58DR11	A2B35DR3 A29B44DR7
320	22/M	CML	BT	44/F	Mother	A24B19DR6 A2BLDR11	A24B19DR6 A29B44DR1
321	2/M	AML	REL	30/M	Brother	A2B51DR11 BLB16DR2	A2B51DR11 BLB37DR4
329	6/M	ALL	REL	33/M	Father	A1B35DR6 A32B18DQ1	A1B35DR6 A30B38DR3
331	27/M	CML	BT	45/F	Mother	A1B38DR7 A2B40DR2	A1B38DR7 A29B44DR5
333	23/M	ALL	REL	19/M	Brother	A26B39DR1 A2B8DR8	A26B39DR1 A3B35DR10
334	51/F	ALL	REL	55/F	Sister	A1B44 A26B7	A1B44 A2B35
401	14/M	AML	REL	44/M	Father	A2B18DR11 A3B51	A2B18DR11 BLB17DR2

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402	29/M	AML	REL	30/M	Brother	A33B12DR7 A30B14DR3	A33B18DR7 A24B14DR1
404	34/M	ALL	REL	30/M	Brother	A1B7DR4 A31B35DR1	A1B7DR4 A32B51
407	13/M	ALL	REL	40/M	Father	A10B14 A2B51	A10B14 A9B35
408	13/M	ALL	REL	40/M	Father	A10B16 A9B18	A10B16 A28B52
409	13/M	ALL	REL	42/M	Father	A11B5DR12 A2B12DR13	A11B5DR12 A9B15DR1

In Table I above the meanings of the various abbreviations are :

AML = acute myeloid leukemia

ALL = acute lymphoblastic leukemia

BT = blastic transformation

REL = relapse

BL = blank

M = male

F = female

Conditioning regimen

All patients received 8 Gy total body irradiation (TBI) in a single dose at a fast dose rate (16 cGy/min midplane) from a 18 MV photon beam linear accelerator on day -5 (5 days prior to engraftment/transplant). Lungs were shielded by individual lead moulds; the corrected mean total lung dose was 7 Gy. Thiotepa (Lederle Laboratories, Pearl River, New York) was administered i.v. on day -4 (4 days prior to engraftment) in two divided doses, 5 mg/kg body weight per dose (4 hours for each infusion, total dose 10 mg/kg body weight). On each day from days -4 to -1 (4 to 1 days prior to engraftment/transplant) rabbit anti-human thymocyte globin (ATG; Fresenius, AG Germany) at a dose of 5 mg/kg body weight was infused over 8 hours, followed by cyclophosphamide (Endoxin-Asta, Asta-Werke, Bielefeld, Germany) administered on days -3 and -2 (3 and 2 days prior to engraftment/transplant) at a dose of 60 mg/kg body weight. No immunosuppressive therapy was given as GvHD prophylaxis following transplant.

On day 0 (i.e. 5 days following the irradiation treatment), bone marrow from a HLA-nonmatched family member, depleted of T-cells by soybean agglutinin and E-rosetting was transplanted into each patient, and preparations of T-cell depleted peripheral blood mononuclear cells (PBMC) from the same donor were administered on days +1 and +2 (i.e. 1 and 2 days after bone marrow transplants; for preparation of the bone marrow and PBMC, see below).

Supportive care

Patients were cared for in laminar air-flow rooms until the neutrophil count recovered to about $1 \times 10^9/L$ (at least $0.5 \times 10^9/L$). All patients received prophylactic trimethoprim-sulfamethoxazole for Pneumocystis carinii, ciprofloxacin for selective gut decontamination, fluconazole for fungal prophylaxis, immunoglobulin (0.5 gr/kg weekly from day -5 to day +90) and total parenteral nutrition. Fever during the period of neutropenia was treated with broad spectrum antibiotics; amphotericin B was added if fever persisted. CMV-prophylaxis consisted of ganciclovir (10 mg/kg/day from day -6 to day -2 and resumed at 5 mg/kg/day from day +7 to day +21 followed by maintenance treatment with 5/mg/kg thrice weekly until day +90). Foscarnet (90 mg/kg/day) was given from day -1 to day +10. All but one patient (UPN 306) received G-CSF (5ug/kg/day) for a mean of 4.7 days (range 2-9) in the immediate post-transplant phase.

All blood products were filtered and irradiated before infusion.

Engraftment and immunological studies

Time to engraftment was assessed by determining the day after transplant on which patients achieved neutrophil count of $0.5 \times 10^9/L$ and a platelet count of $2.5 \times 10^9/L$ independent of transfusion support. Chimerism was assessed by karyotyping of peripheral blood lymphocytes and the analysis of restriction fragment length polymorphism (RFLP) in both peripheral blood and bone marrow (Yam, P.Y. et al., 1987). The degree of acute GVHD was assessed using standard clinical criteria (Glucksberg, H. et al., 1974).

Post-transplant lymphoid cell subsets were identified by two-color immunofluorescence and flowcytometry (Velardi, A. et al., 1988a). Cytotoxicity against a panel of NK cell-sensitive and resistant targets was evaluated by a $^{51}\text{-Cr}$ release assay as described (Velardi, A. et al., 1990). T cell proliferation was assessed by stimulating cells with anti-CD3 MoAb, and pulsing the cultures with ^3H -thymidine for 12 hours at the end of a 72-hour culture period. For detection of IL-2 activity in culture supernatants, the CTLL murine cell line assay was utilized (Velardi, A. et al., 1988b).

Bone marrow and peripheral blood mononuclear cells collection

Donor bone marrow cells were obtained under general anesthesia by multiple aspirations from the iliac crests bilaterally and cryopreserved, as discussed below. Recombinant human G-CSF (rhG-CSF; 12 ug/kg/day) was administered to donors by continuous subcutaneous infusion from 24 hours after bone marrow harvesting and continued for 5-7 days. Two to four leukaphereses were performed between days 4 and 7 in the first 7 donors, who received rhG-CSF for 6-7 days; while the remaining 10 donors, who were treated with rhG-CSF for 5-6 days, underwent two-three leukaphereses (days 4, 5 and 6).

The donors reported no untoward effects during or following the administration of rhG-CSF or from the blood collections.

Bone marrow and peripheral blood mononuclear cell processing

All bone marrow preparations were depleted of T lymphocytes using the soybean agglutination and E-rosetting technique, as previously described (Reisner, Y. et al., 1986). This procedure results in a 3-3.5 log₁₀ reduction in the number of clonable T lymphocytes. Depletion of T lymphocytes from the peripheral blood mononuclear cells in the first seven cases, was achieved by a two step E-rosetting procedure, whereas the combined soybean agglutination and E-rosetting technique was used in the last ten cases. Aliquots were taken for differential cell counts, monoclonal antibody (MoAb) staining and GFU-GM assay at each stage of processing. T cell-depleted marrow and peripheral blood cells were frozen in a controlled rate liquid nitrogen freezer and stored in the vapor phase of liquid nitrogen. In some cases, the collections from peripheral blood were performed on the day before and on the day of the transplant; these cells were not cryopreserved.

CFU-GM were measured in whole blood and in the leukapheresis product by plating 0.5×10^5 mononuclear cells in a 3% agar solution containing 10% of 5637 cell-line conditioned medium, 20% fetal bovine serum and Iscove medium. Colonies of greater than 40 cells were counted on an inverted microscope (Leica, Wetzlar, Germany) after 10-14 days.

The number of CD34+ cells were measured both in whole blood and in the leukapheresis product with a direct immunofluorescence technique using the fluorescein conjugate HPCA-2 monoclonal antibody (Becton Dickinson, Palo Alto, CA). Negative control was assessed using a mouse IgG1-FITC. Cells were analyzed on a Profile II (Coulter Corporation, Hialeah, FL). A gate was established to include only lymphocytes and

mononuclear cells. 10,000 cells were evaluated.

The T lymphocytes before and after T cell-depletion were evaluated with an immunocytological technique using an anti-CD3 monoclonal antibody as previously described (Cordell, J.L. et al., 1984).

Results

Mobilization and collection of peripheral blood progenitor cells (PBPC)

In the normal donors, under steady state hematopoiesis (baseline), minimal amounts of circulating CD34+ cells (median 0.5/uL blood; range 0 to 15/uL) and CFU-GM (median 122,7/ml; range 0 to 268) were detectable. With G-CSF treatment donor white cell count rose from a median of $6.7 \times 10^9/L$ to $72 \times 10^9/L$ by day 7.

Peak levels of CD34+ cells, as well as CFU-GM, were reached simultaneously at a median of 5 days. Median values of peak levels were 830.7 CD34+cell/uL (range 92.8 - 1,035) and 12,347 CFU-GM/ml (range 549 - 20,126).

The 2-4 leukapheresis procedures yielded a mean total number of 10.4×10^8 mononuclear cells per kilogram donor body weight (range $4-23 \times 10^8$). The combined leukapheresis products contained a mean of $11.62 \pm 4.74 \times 10^6/kg$ CD34+cells (range 5.47-18.99) and $73.182 \pm 40.8 \times 10^4/kg$ CFU-GM (range 13 - 132.53).

T cell-depletion of bone marrow and peripheral blood mononuclear cells

Table II lists the mean number (kg/body weight) of mononuclear cells, CFU-GM, CD34+ and CD3+ cells present in bone marrow, peripheral blood and the combined products given to the patients following T cell-depletion.

The median dose of T cells infused was higher in the first seven patients (group I) who received E-rosette-depleted peripheral blood mononuclear cells (PBMC) than in those (group II) whose leukapheresis product was depleted of T lymphocytes by the combined soybean agglutination (SBA) and E-rosetting technique. In both groups the average concentration of CFU-GM in the combined product was 7-10 fold greater than that found in bone marrow alone.

Table II

**Characteristics of Transplanted Bone Marrow and
Peripheral Blood Cells after T Cell-Depletion**

	GROUP I			GROUP II		
	BM	PBMC	TOTAL	BM	PBMC	TOTAL
MNC ($\times 10^8/\text{kg}$) ^a	0.31	5.96	6.27	0.27	2.98	3.25
CFU-GM ($\times 10^4/\text{kg}$)	12.77	71.55	84.32	4.56	35.80	40.36
CD34+ ($\times 10^6/\text{kg}$)	1.9	12	13.9	N.D.	16	16
CD3+ ($\times 10^5/\text{kg}$)	0.32	5.91	6.23	0.19	1.24	1.43

^a recipient body weight

Group I = 7 donors. BM was T cell-depleted by SBA and 1 step E-rosette. PBMC were T cell-depleted by only 2 step E-rosette. Donors underwent 2-4 leukaphereses.

Group II = 10 donors. BM and PBMC were T cell-depleted by SBA + 2 step E-rosette. Donors underwent 2-3 leukaphereses.

Engraftment

One patient (UPN331) rejected the graft on the 18th post-transplant day, after initial myeloid engraftment. RFLP analysis of granulocytes confirmed that they were donor-derived on day 14 (data not shown). This early rejection was associated with the abrupt emergence of host T cells that exhibited donor specific cytotoxic reactivity.

The other 16 patients had early and sustained engraftment. They achieved peripheral blood neutrophil counts over $0.5 \times 10^9/\text{L}$ and over $1.0 \times 10^9/\text{L}$ at a mean of 10.2 days (range 9-17) and 11.5 days (range 10-22), respectively. Platelet counts of $25 \times 10^9/\text{L}$ and $50 \times 10^9/\text{L}$

were reached at a mean of 17.2 days (range 10-29) and 30 days (range 14-60), respectively. The time course of engraftment is illustrated in Fig. 1. The curves represent the time required for T cell-depleted "three loci" incompatible transplants according to the present invention, to reach 0.5 and 1.0×10^9 /neutrophils/L, as compared with our own historical control group of 23 patients who received autologous chemotherapy/cytokine-mobilized PBPCs (data not shown) and 93 patients transplanted with T cell-depleted HLA genotypically identical bone marrows (data not shown). From this comparison it was observed that the T cell-depleted incompatible transplants of the present invention enabled a more rapid obtention of these amounts of neutrophils in essentially all of the patients examined.

RFLP analysis documented full donor type chimerism in both peripheral blood and bone marrow of the 16 engrafted patients (data not shown).

Immune reconstitution

Phenotypic and functional analyses of post-transplant lymphocyte subsets were performed and compared with those obtained in HLA-matched T cell-depleted BMT recipients (data not shown). Whereas essentially identical data were obtained for B-cell and T-cell subsets in the two BMT settings, a two-fold increase in the early (1-2 months post-grafting) natural killer cell wave was noted in mismatched, i.e. the present invention, as compared to matched, transplants. The number of CD56+/CD16+/CD3-, NK cells reached a peak value of 707 ± 212 ul after mismatched BMT and 306 ± 19 after matched BMT.

Graft versus host disease

One patient (UPN317) developed grade IV acute GVHD which was fatal. It is worth noting that she received a greater quantity of T lymphocytes (11.30×10^5 /kg) than any of the other patients. There were no other cases of acute GVHD \geq grade II.

Toxicity and clinical outcome

In almost all patients, thiotepa caused a sunburn-like erythema, that gradually faded and peeled-off; mild reversible oral mucositis developed in all patients. Mild diarrhea was generally seen within 2-4 days of completion of the conditioning regimen and resolved spontaneously. Transient hemorrhagic cystitis complicated the course of four patients and resolved with hydration and continuous bladder irrigation. Moderate veno occlusive disease (VOD) of the liver occurred in two patients; their bilirubin levels ranged from 2 to 7 mg/dl and returned to

normal in ten days with sodium restriction and diuretics. The median time to onset of VOD was 6 days post-transplant.

Six patients developed interstitial pneumonitis between days +14 and +160 and died from respiratory failure (Table III). No infectious cause could be identified in two (UPN319, 404), while CMV was the causative agent in four (UPN315, 320, 321, 334). Hematological remission and full donor type chimerism was documented in all 6 cases at the time of death. CMV-related gastroenteritis occurred in 5 patients but resolved with ganciclovir treatment.

The one patient (UPN331) who experienced graft failure and the one patient with GVHD, died. One (UPN 401) died from fungal infection. There have been two relapses, both in patients transplanted for ALL, within 2 months from the transplant..

Six patients are alive and well at a median follow-up of 230 days (range 100-485) post-transplant, all with a Karnofsky performance status of 100%.

The above clinical outcome of the present study is summarized in Table III.

TABLE III
Clinical Outcome

UPN	Disease	Status at transplant	Blasts (%) in bone marrow	Engraftment	A-GvHD (grade)	Current Status (June 30, 1994)
306	AML	2nd Relapse	80	YES	0	Alive in CCR on Day +485
313	AML	Induction Failure	100	YES	0	Alive in CCR on Day +413
315	ALL	3rd Relapse	100	YES	0	Died on Day 120 from CMV-IP
317	ALL	2nd Relapse	100	YES	IV	Died on Day 60 from GvHD
319	CML	2nd Blast crisis	80	YES	0	Died on Day 90 from Idiopat-IP
320	CML	3rd Blast crisis	16	YES	0	Died on Day 20 from CMV-IP
321	AML	3rd Relapse	100	YES	0	Died on Day 18 from CMV-IP
329	ALL	3rd Relapse	100	YES	0	Relapsed on Day 60, Died on Day 70
331	CML	2nd Blast crisis	30	NO	N.E.	Died on Day 45 from sepsis
333	ALL	3rd Relapse	15	YES	0	Relapsed on Day 50, Died on Day 60
334	ALL	2nd Relapse	15	YES	I	Died on Day 180 from Idiopat-IP
401	AML	3rd Relapse	100	YES	0	Died on Day 45 from sepsis

			17			
402	AML	Induction Failure	100	YES	I	Alive in CCR on Day +157
404	ALL	2nd Relapse	100	YES	0	Died on Day 62 from Idiopat-IP
407	ALL	2nd Relapse	15	YES	I	Alive in CCR on Day +126
408	ALL	3rd Relapse	15	YES	I	Alive in CCR at Day +110
409	ALL	3rd Relapse	10	YES	I	Alive in CCR on Day +100

The abbreviations in Table III above are the same as in Table I, with the following additions :

N.E. = Not Evaluable

CCR = Continuous Complete Remission

IP = Interstitial pneumonitis

Thus, in our study a 7-10 fold increase in the dose of the transplant inoculum was achieved by adding T cell-depleted rhG-CSF mobilized PBPCs to the T cell-depleted bone marrow.

The very large cell dose we infused after the intensive conditioning regimen were followed by prompt and sustained engraftment in 16 of 17 recipients of haploidentical "three loci" mismatched T cell-depleted bone marrow. Neutrophil and platelet recovery was very rapid and the engraftment characteristics were very similar to those observed in syngeneic PBMC transplants or in our historical control group of patients who received autologous chemotherapy/rhG-CSF-mobilized PBPCs.

The impressive rate of engraftment across the most difficult histoincompatibility barrier demonstrate that in humans, as in mice, the stem cell dose plays a critical role in the engraftment of T cell-depleted transplants. This concept is further supported by the finding that the same pre-transplant conditioning failed to promote engraftment in any of the five patients transplanted with conventional doses of T cell-depleted "three loci" mismatched bone-marrow (our unpublished observations).

One potential major concern raised by the use of a large T cell-depleted inoculum is an increased risk of GVHD, mainly due to T cell contamination of PBPCs. However, greater than grade I GVHD was extremely rare and occurred in only one of the evaluable patients who received the largest number of T cells ($11.3 \times 10^5/\text{kg}$, almost two fold more than the average numbers administered to group I and about ten fold more than the average of group II), greater than $2 \times 10^5/\text{kg}$ which is considered the threshold dose of clonable T cells which leads to GVHD. However, it is likely that ATG, given between days -5 and -2, contributes to lowering both the frequency and severity of GVHD by exerting a cytotoxic effect against donor inoculum T lymphocytes.

Of more concern is the question of CMV disease. CD8+ CMV-specific cytotoxic T lymphocytes (CTL) are responsible for protective immunity and elimination of active infection. As CMV-specific CTL responses may require an extended time period after mismatched BMT and they are HLA-restricted, susceptibility for CMV-infections is greater with mismatched than matched BMT. The rapid hematopoietic reconstitution observed in our series of patients, should permit early prophylaxis of CMV infections with ganciclovir and so contribute to lowering mortality. After the schedule of ganciclovir was modified to begin day +7, no case of CMV-pneumonia was documented in the 9 patients (see group II patients, Tables II and III).

Example 2 : Engraftment of T cell-depleted transplants in sublethally irradiated recipients

In Example 1, the engraftment of the T cell-depleted bone marrow and PBMC transplants was studied in patients who had all received lethal doses of irradiation. Based on the encouraging (6 living, apparently healthy patients) results of this study, we extended this approach to determine whether in sub-lethally irradiated recipients engraftment of such bone marrow and PBMC transplants could also occur successfully, without the need for post-transplant immune suppression.

Using an accepted animal (mouse) model, we tested the feasibility of such an approach for human applications, according to the following treatment protocol : C3H/HeJ mice were conditioned by a single dose TBI in the range of 6-8 Gy and then transplanted with increasing doses of T-cell depleted bone marrow (BM) from C57BL/6 donors. Donor type chimerism determination one month post-transplant revealed that while engraftment was effective in mice conditioned with 8Gy upon transplantation of 8×10^6 cells, it could also be generated following conditioning with a sublethal dose of 6.5 Gy TBI (survival in untransplanted mice >90%),

provided that the BM dose was increased by about 4 fold. Thus, transplantation of 40×10^6 , 20×10^6 and 10×10^6 cells led to donor type chimera in 18/21, 13/26 and 1/26 of the recipients, respectively.

Transplantation of T-cell depleted BM of C57BL/6-nude donors necessitated 200×10^6 cells to achieve engraftment, but repletion of the BM with 5×10^4 purified thymocytes (using peanut agglutinin, PNA) reduced the minimal number required to achieve donor type chimeras to 50×10^6 cells, similar to the results with normal C57BL/6 marrow.

These results suggest that the new source of G-CSF mobilized human hematopoietic pluripotential stem cells (see Example 1) which can provide at least one log more of such cells, compared to the bone marrow alone, may extend the use of mismatched bone marrow transplants to patients with non-malignant diseases for whom supralethal or lethal conditioning is not a prerequisite, and thus a sublethal conditioning is suitable. Examples of such diseases are SCID, osteopetrosis, aplastic anemia, Gaucher's disease, thalassemia and other congenital or genetically-determined hematopoietic abnormalities, or prior to transplantation of another organ from the same donor to a same recipient.

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CLAIMS

1. A method for bone marrow transplantation to a patient in need thereof which comprises :
 - i) conditioning the host patient under sublethal, lethal or supralethal conditions; and
 - ii) transplanting to the conditioned host patient a high dose of stem cells which is at least about 3-fold greater than conventional doses used in T cell-depleted bone marrow transplantation.
2. A method according to claim 1, wherein the conditioned patient is transplanted with high doses of T-cell-depleted bone marrow cells and T-cell-depleted stem cell-enriched peripheral blood cells from an HLA-nonmatched donor.
3. A method according to claim 1 or 2, wherein T cell-depletion is carried out by soybean agglutinin and E-rosetting with sheep red blood cells and the dose of CD34+ stem cells used is at least about 5.5×10^6 /kg body weight of the host patient
4. A method according to any one of claims 1 to 3 wherein the donor is a close relative of the patient.
5. A method according to any one of claims 2 to 4 wherein the T cell-depleted stem cell-enriched peripheral blood cells are obtained by leukapheresis of peripheral blood from the donor after stimulation by a suitable cytokine.
6. A method according to claim 5 wherein the donor is stimulated with granulocyte colony-stimulating factor (G-CSF).
7. A method according to any one of claims 1 to 6 wherein the patient is conditioned under lethal or supralethal conditions.
8. A method according to claim 7 wherein said lethal or supralethal conditions include total body irradiation (TBI).

9. A method according to claim 8 wherein a single or fractionated irradiation dose within the range of 7-16 Gy TBI is used.
10. A method according to claim 9 wherein the host patient receives a 8 Gy single dose TBI.
11. A method according to any one of claims 8 to 10 wherein the host patient is conditioned by TBI followed by treatment with myeloablative and immunosuppressive agents.
12. A method according to claim 7 wherein said lethal or supralethal conditions include treatment with myeloablative and immunosuppressive agents without TBI.
13. A method according to claim 11 or 12 wherein the myeloablative agent is selected from busulphan, dimethyl myleran and thiotepa, and the immunosuppressive agent is selected from prednisone, methyl prednisolone, azathioprine, cyclophosphamide, cyclosporine, monoclonal antibodies against T cells, antilymphocyte globulin and antithymocyte globulin.
14. A method according to claim 11 wherein the myeloablative agent is thiotepa and the immunosuppressive agents are antithymocyte globulin and cyclophosphamide.
15. A method according to claim 12 wherein the myeloablative drug busulphan is administered to the patient from day -8 to day -5 prior to transplant at a daily dose of 2-5 mg/kg body weight for lethal conditions and above 5 mg/kg body weight for supralethal conditions, and this treatment is followed by administration of the immunosuppressive drug cyclophosphamide from day -4 to day -1 prior to transplant at a daily dose of 50 mg/kg body weight.
16. A method according to any one of claims 1 to 15 for the treatment of patients afflicted with a malignant or non-malignant disease.
17. A method according to claim 16 for the treatment of malignant diseases.

18. A method according to claim 17 for the treatment of acute lymphoblastic leukemia (ALL), acute myelocytic leukemia (AML) or chronic myelocytic leukemia (CML).
19. A method for treatment of a leukemia patient which comprises :
 - i) conditioning the leukemia patient under lethal or supralethal conditions with TBI, antithymocyte globulin (ATG), thiotepa and cyclophosphamide; and
 - ii) transplanting to the conditioned patient an average of about 0.31×10^8 /kg T-cell-depleted bone marrow cells and an average of about 6×10^8 /kg T-cell-depleted peripheral blood cells from an HLA-nonmatched family member who was prior stimulated with G-CSF.
20. A method according to any one of claims 1 to 6 wherein the patient is conditioned under sublethal conditions.
21. A method according to claim 20 wherein said sublethal conditions include TBI.
22. A method according to claim 21 wherein a single or fractionated irradiation dose within the range of 3-7 Gy TBI is used.
23. A method according to claim 22, wherein a single or fractionated dose of about 6.5 Gy TBI is used.
24. A method according to any one of claims 21 to 23, wherein the host patient is conditioned by TBI followed by treatment with myeloablative and immunosuppressive agents.
25. A method according to claim 20 wherein said sublethal conditions include treatment with myeloablative and immunosuppressive agents without TBI.
26. A method according to claim 24 or 25 wherein the myeloblative agent is selected from busulphan, dimethyl myleran and thiotepa, and the immunosuppressive agent is selected from prednisone, methyl prednisolone, azathioprine, cyclophosphamide, cyclosparine, monoclonal antibodies against T cells, antilymphocyte globulin and antithymocyte globulin.

27. A method according to claim 25 or 26 wherein the myeloablative drug busulphan is administered to the patient from day -8 to day -5 prior to transplant at a daily dose of 1-2 mg/kg, and this treatment is followed by administration of the immunosuppressive drug cyclophosphamide from day -4 to day -1 prior to transplant at a daily dose of 50 mg/kg body weight.

28. A method according to any one of claims 1 to 6 and 20 to 27 for the treatment of a non-malignant disease.

29. A method according to claim 28 for the treatment of aplastic anemia, osteopetrosis, severe combined immunodeficiency syndromes (SCID), Gaucher's disease, thalassemia and other forms of congenital or genetically-determined hematopoietic abnormalities, or for bone marrow transplantation prior to transplantation of an organ from the same donor to the same recipient.

1/1

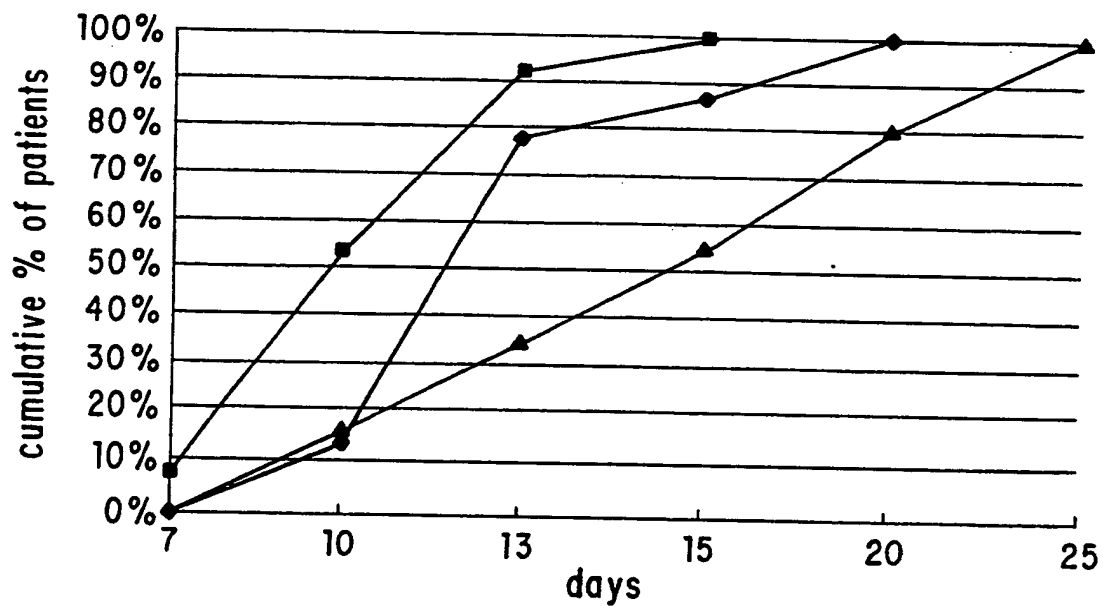


FIG. 1A

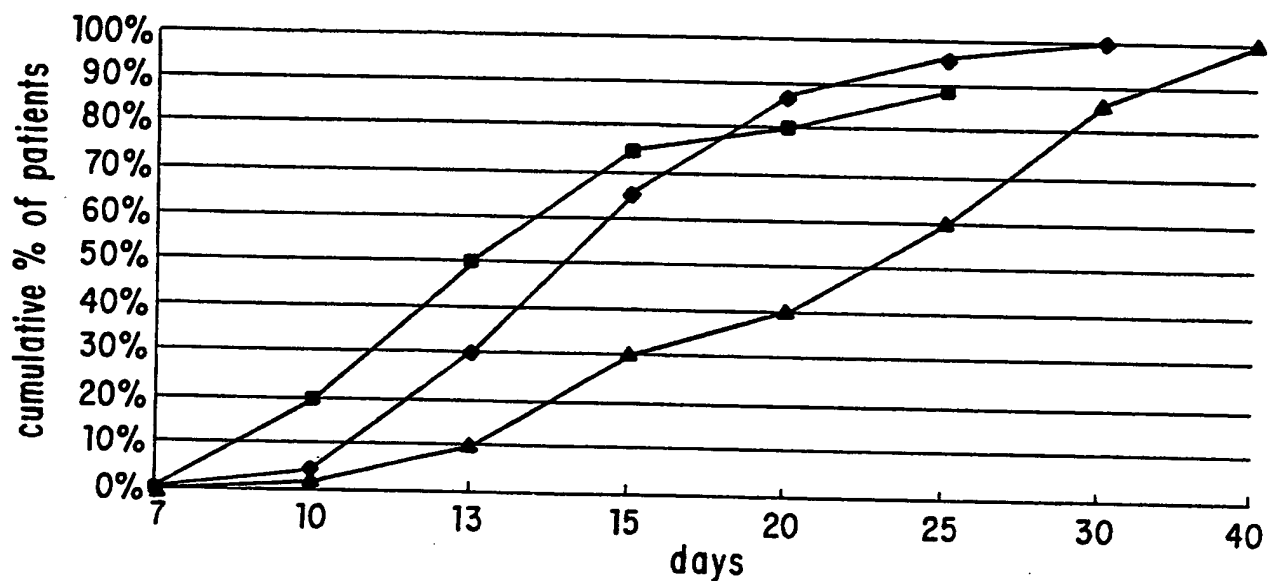


FIG. 1B

- Present study
- ▲— Autologous chemotherapy/cytokine-mobilized PBPCs transplant
- ◆— HLA identical T cell-depleted BMT

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12610

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/12, 35/28, 38/27

US CL :424/534, 140.1, 85.1; 604/4, 6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/534, 140.1, 85.1; 604/4, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Volume 81, Number 7, issued 01 April 1993, Mangan et al., "Serum interleukin-3 levels following autologous or allogeneic bone marrow transplantation: effects of T-cell depletion, blood stem cell infusion, and hematopoietic growth factor treatment", pages 1915-1922, see abstract.	1-29
Y	Blood, Volume 81, Number 7, issued 01 April 1993, Neben et al., "Mobilization of hematopoietic stem and progenitor cell subpopulations from the marrow to the blood of mice following cyclophosphamide and/or granulocyte colony-stimulating factor", pages 1960-1967, see abstract.	1-29

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
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P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 FEBRUARY 1995

Date of mailing of the international search report

02 MAR 1995

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Haematologica, Volume 78, Number 1, issued 1993, Zinzani et al. "Granulocyte colony stimulating factor G-CSF as adjunct therapy in relapsed-resistant high-grade non-Hodgkin's lymphoma", pages 40-43, see abstract.	1-29
Y	Cancer Research, Volume 45, Number 9, issued 1985, Reisner et al. "Differential binding of soybean agglutinin to human neuroblastoma cell lines potential application to autologous bone marrow transplantation", pages 4026-4031, see abstract.	1-29